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International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/87, A01K 67/027, C12N 15/00</b> <b>// A61K 47/48</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 95/01448</b> <b>(43) International Publication Date:</b> 12 January 1995 (12.01.95)
<b>(21) International Application Number:</b> PCT/US94/06440 <b>(22) International Filing Date:</b> 15 June 1994 (15.06.94)  <b>(30) Priority Data:</b> 08/085,696      30 June 1993 (30.06.93)      US  <b>(71) Applicant:</b> DCV BIOLOGICS L.P. [US/US]; 3411 Silverside Road, Quillen Building, Wilmington, DE 19810 (US).  <b>(72) Inventors:</b> FITZPATRICK-McELLIGOTT, Sandra, G.; 27 Long Point Lane, Rose Valley, PA 19063 (US). LAVIN, John, Gerard; 15 Wellesley Road, Swarthmore, PA 19081-1232 (US). RIVARD, Germain, F.; 1644 Lombard Street, Philadelphia, PA 19146 (US). SUBRAMONEY, Shekhar; 425 Stella Drive, Hockessin, DE 19707-1922 (US).  <b>(74) Agents:</b> CHRISTENBURY, Lynne, M. et al.; E.I. du Pont de Nemours and Company, Legal/Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).		<b>(81) Designated States:</b> AU, CA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> A METHOD FOR INTRODUCING A BIOLOGICAL SUBSTANCE INTO A TARGET  <b>(57) Abstract</b>  A method for introducing a biological substance into a target which utilizes particles having a substantially pure carbonaceous surface to which is associated a biological substance wherein the particles are sufficiently small to penetrate the target without killing the target is described.		



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TITLE

A Method for Introducing a Biological Substance Into a Target

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FIELD OF THE INVENTION

This invention concerns a method for introducing a biological substance into a target and, in particular, to a method which utilizes particles having a substantially pure carbonaceous surface to which is associated a biological substance wherein the particles are sufficiently small to penetrate the target without killing the target.

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BACKGROUND OF THE INVENTION

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Biological delivery systems have long been the focus of much research due to the intense interest in introducing a variety of biological substances into a target. Such substances include nucleic acids, genetic constructs, drugs, hormones, proteins, biological stains, etc. Targets include cells, germ cells, plants, microbes, animals, organelles, tissues, etc. Diverse applications for biological delivery systems are known as is illustrated in The Pharmacological Basis of Therapeutics, Goodman and Gilman, Chapter 1, pages 1-28 (1970).

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A number of delivery systems for biological substances are available. Among those options are oral ingestion, nasal sprays, pulmonary absorption, parenteral injection, transdermal or topical delivery. However, there is a desire to extend the accessibility and release (bioavailability) of a biological substance to its target. For example, genetic material can be delivered into a target. Existing technologies for introducing genetic material into living cells involve a number of systems some of which utilize biological vectors such as retroviruses or Agrobacterium to accomplish gene transfer. Others rely on chemical,

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e.g., calcium phosphate precipitation, polyethylene glycol, lipofection, or liposomes or physical means such as injection, microinjection or electroporation or particle bombardment to facilitate DNA transfer across cell membranes. These technologies are reviewed in Klein et al., Bio/Technology, 10: 286-291 (March 1992).

Particle bombardment also referred to as Biolistic® is a physical method for gene delivery which involves accelerating DNA-coated particles (microprojectiles) directly into cells. The Biolistic® process is described in U.S. Patent No. 4,945,050 which issued to Sanford et al. on July 31, 1990; Sanford, Biomedical Engineering: Opening New Doors, Proceedings of the 1990 Biomedical Engineering Society, Milurlecky and Clarke (Eds.) (1990); Klein et al., Bio/Technology, 10: 286-291 (March 1992). These microprojectiles must have sufficient mass to acquire the necessary kinetic energy to penetrate the particular cell sought to be penetrated and they must have integrity sufficient to withstand the physical forces inherent in the the process. The microprojectiles are described as being inert particles including ferrite crystals, gold, tungsten, spheres as well as particles of low density such as glass, polystyrene and latex beads. Biological particles can also be used. The biological substance to be transported is coated, impregnated or otherwise operably associated with solid particle to be introduced into the cell.

Particle-mediated transformation of somatic cells is described in PCT International Application having International Publication Number WO91/0035 published on January 10, 1991. In this process an electric discharge particle acceleration apparatus such as the Accell® particle acceleration apparatus is used to inject very small particles of gold or other dense material carrying genetic constructs coated on them into the living cells of animals.

15           One alternative considered by researchers to  
overcome this problem is to exploit the ability of  
spermatozoa to introduce foreign DNA into eggs at  
fertilization. Lavitrano et al., Cell 57: 717-723  
(1989) purported that they were able to use sperm to  
20 transfer plasmid DNA into eggs. However, several  
attempts to duplicate this work have been unsuccessful  
to date, Brinster et al., Cell 59: 239 (1989).

35 Recently, particles having a substantially pure carbonaceous surface were discovered in 1991 as a by-product of fullerene production, Iijima, Nature, Vol. 259, pages 56-58 (1991). Most recently, Ruoff et al.,

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Science, Vol. 259, pages 346-348 (January 15, 1993) have described novel particles in which single crystal metals were encapsulated in particles having a substantially pure carbonaceous surface. Specifically, single domain  
5  $\alpha$ -LaC<sub>2</sub> small crystals were encapsulated inside particles having a substantially pure carbonaceous surface. To date no applications have been reported for these particles having a substantially pure carbonaceous surface and the like.

10 Surprisingly and unexpectedly, it has been found that such particles can be used not only to transform targets by means such as particle bombardment, but also such particles can be used to deliver a biological substance into a target as part of a  
15 biological delivery system. Such findings have not heretofore been reported.

#### SUMMARY OF THE INVENTION

This invention concerns a method for  
20 introducing a biological substance into a target which comprises  
(a) associating the biological substance with a particle having a substantially pure carbonaceous surface, said particle having a diameter sufficiently  
25 small to penetrate a target without killing the target, and

(b) inserting the particle of step (a) into the target.

In another embodiment this invention concerns  
30 a method for introducing exogenous nucleic acid into sperm which comprises

(a) accelerating a particle having a substantially pure carbonaceous surface and to which surface is associated the exogenous nucleic acid,  
35 wherein said particle encapsulates a magnetic core said particle having a diameter sufficiently small to penetrate the sperm without killing the sperm, and

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propelling said particles at the sperm whereby said particles penetrate the sperm; and

(b) magnetically selecting the sperm into which the particles have penetrated.

5 In still another embodiment this invention concerns a method for making a transgenic animal or non-human mammal which comprises

(a) accelerating a particle having a substantially pure carbonaceous surface and to which  
10 surface is associated the exogenous nucleic acid, wherein said particle encapsulates a magnetic core said particle having a diameter sufficiently small to penetrate the sperm without killing the sperm, and propelling said particles at the sperm whereby said  
15 particles penetrate the sperm;

(b) magnetically selecting the sperm into which the particles have penetrated;

(c) fertilizing an egg with said magnetically selected sperm in vivo or in vitro;

20 (d) allowing the product of step (c) to develop to term;

wherein the product of step (d) and/or its progeny is capable of expressing the exogenous nucleic acid.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a high resolution electron microscopic image of particles having a substantially pure carbonaceous surface produced by arc-discharge.

30 Figure 2 depicts a high resolution electron microscopic image of a particle having a substantially pure carbonaceous surface encapsulating a dense core of  $\alpha$ -lanthanum dicarbide.

Figure 3 depicts a high resolution electron  
35 microscopic image of a particle having substantially pure carbonaceous surface encapsulating dense magnetic material, specifically,  $\alpha$ -gadolinium dicarbide.

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Figure 4 is a schematic of a particle, either magnetic material or inert material coated with magnetic material, encapsulated in a particle having a substantially pure carbonaceous surface by chemical vapor deposition of carbon.

Figure 5 is a schematic of an apparatus for conducting arc-discharge experiments to produce particles having a substantially pure carbonaceous surface.

Figure 6 is a low resolution electron microscopic image of a product of an arc-discharge experiment.

Figure 7 depicts particles having a substantially pure carbonaceous surface encapsulating  $\alpha$ -gadolinium dicarbide to which have been associated fluorescent DNA.

Figure 8 depicts gene expression in an animal ovary cell line after particle bombardment with particles having substantially pure carbonaceous surface and encapsulating  $\alpha$ -gadolinium dicarbide to which has been associated plasmid DNA containing the *lac z* coding region.

Figure 9 depicts bovine sperm containing particles having a substantially pure carbonaceous surface and encapsulating  $\alpha$ -gadolinium dicarbide to which has been associated fluorescently-labelled DNA. The particles were inserted using particle bombardment.

#### DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise indicated, all references discussed herein are incorporated by reference.

The method of the present invention utilizes novel particles having a substantially pure carbonaceous surface to which is associated a biological substance. The particles have a diameter sufficiently small to penetrate a target without killing the target. Insertion of the particles into the target can be



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accomplished using any of a variety of techniques well known to those skilled in the art.

The term "carbonaceous" as used herein means composed of in part, or entirely, of carbon.

5       The term "substantially pure carbonaceous surface" as used herein means that the outer layers of the particle have an amount of carbon necessary to which a biological substance can associate and render the particle compatible with the target. Preferably the  
10 outer layers of the particle are greater than about 90% carbon. More preferably, the outer layers of the particle are greater than about 95% carbon. However, it is also possible that a non-carbonaceous component or components might be present in the outer layer provided  
15 that they do not interfere with association of a biological substance to the particle and they do not render the particle incompatible with the target. The substantially pure carbonaceous surface facilitates the association of a greater quantity of biological material  
20 to the carbonaceous surface. This in turn can help to increase bioavailability of the biological substance to the target.

Any particles having a substantially pure carbonaceous surface as defined herein can be used to  
25 practice the invention. Examples of suitable particles include, but are not limited to, those classified as particles having a substantially pure carbonaceous surface, particles having a substantially pure carbonaceous surface and encapsulating a dense core  
30 which can be either non-magnetic or magnetic, or fullerites (crystalline fullerenes, typically C<sub>60</sub> - C<sub>70</sub>) of the appropriate particle size as described in Prassides et al., Carbon, Vol. 30, pages 1277-1286 (1992).

35       One important aspect of these particles is that they have a substantially pure carbonaceous surface to which a biological substance can be associated. The

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substantially pure carbonaceous surface serves not only as a site to which a biological substance can be associated but it also renders the particles compatible with a wide variety of environments depending upon the nature of the target because the inert, non-toxic coating is compatible with the cellular milieu. In addition, biological substances including nucleic acids can be associated effectively onto the surface which in turn improves delivery of the biological substance to the target.

The size of the particles should be a size substantially smaller than that of the target sought to be penetrated. It should be small enough to penetrate the target and become incorporated into the target without killing the target. For example, the particles can be very small in the nanometer range from about 5 nm to 100 nm. However, if the particles aggregate, the aggregates can be bigger than this. For example, aggregates can range in size from about 10 nm to about 1.0 micron. Preferably, the size of the particles and any aggregate of the particles which might form will be in the range from about 5 nm to about 1.0 micron. It has been found that aggregates having sizes ranging from about 10 nm to about 1.0 micron are useful in practicing the methods of the invention.

Depending upon the means used to insert the particles into the target, it may also be necessary to consider whether it has the ability to impart sufficient kinetic energy to penetrate the desired target. When this is a consideration, the optimum particle size is small enough to produce minimal cell damage and large enough to acquire sufficient momentum, to penetrate the target; momentum being a function of size, density and velocity.

Particles having a substantially pure carbonaceous surface can be made in a variety of ways. For example, they can be made by an arc process as

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described in Iijima, Nature, vol. 354, pages 56-58 (1991) or chemical vapor deposition. These processes are further illustrated in the examples below. Such particles can be polyhedral or tubular in morphology having a hollow core. The density of these particles can be measured by density gradient tubes (ASTM D150556-68). The density usually exceeds about 2.0 gm/cc. Figure 1 depicts such a particle.

Particles having a substantially pure carbonaceous surface and which encapsulate a dense core are similar to the particles having a substantially pure carbonaceous surface described above except that it has a dense core. Any dense material whether non-magnetic or magnetic can be used as the dense core long as it is heavier than carbon and can withstand the encapsulation process. Examples of suitable dense cores include heavy elements such as lanthanum, yttrium, copper or their carbides. An example of an  $\alpha$ -lanthanum dicarbide encapsulated particle having a substantially pure carbonaceous surface is depicted in Figure 2. It shows a particle having a substantially pure carbonaceous surface (1) encapsulating an  $\alpha$ -lanthanum dicarbide crystal (2) as well as an empty region inside the particle (3). Depending upon the process used to make the particle it is possible that an empty region may not be present.

Preferably, these particles having a substantially pure carbonaceous surface encapsulate a magnetic core in the form of a metal or carbide. They are similar to the particles described above except that the dense core is magnetic. Any magnetic material can be used to fill the core either partially or entirely. There can be mentioned as one example the ferromagnetic elements iron, cobalt, nickel, or gadolinium. For example,  $\alpha$ -gadolinium dicarbide can be used. Figure 3 shows a particle having a substantially pure carbonaceous surface (1) encapsulating an  $\alpha$ -gadolinium

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Examples of biological substances which can be associated with the particles having a substantially pure carbonaceous surface include, but are not limited to, nucleic acids, genetic constructs, proteins such as enzymes, toxins, pharmaceutical compounds, viruses, hormones, lipids, biological stains, organelles, and vesicles. Preferably, the genetic construct should code for a protein with effective flanking regulatory sequences to express the protein in the target. It is also possible to use a genetic construct which is an RNA

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strand or DNA sequence effective to inhibit a native gene or to retard a disease process. DNA or RNA sequences and their derivatives which inhibit gene expression can also be referred to as antisense.

5           Examples of targets into which the particles can be inserted include, but are not limited to, cells, germ cells such as sperm. There can also be mentioned microbes, microalgae, plants, organelles, cells, animals, organoids, organs and tissues. "Organoids" are  
10 organ-like structures of clusters of cells or tissues which can be created in an in vitro culture and surgically re-implanted into a living animal. Such organoid cultures may be used effectively with mammals, and in humans to reintroduce transformed somatic cells  
15 back into a patient for genetic therapy or other therapeutic use. Examples of microbes include bacteria, fungi, viruses, etc. Examples of microalgae include chlorella, chlamydomonas, etc. Examples of organelles include nucleic, mitochondria, chloroplasts,  
20 etc.

The particles having a substantially pure carbonaceous surface to which is associated a biological substance can be inserted into a target using any number of means available to those skilled in the art. There  
25 can be mentioned direct parenteral injection such as intramuscular, intravenous and subcutaneous. There can also be mentioned nasal sprays and implants as well as microinjection.

The preferred means by which to insert these  
30 particles into a target is by particle bombardment also referred to as Biolistic®. This technique involves accelerating particles to which is associated a biological substance directly into a target. Particle acceleration constitutes the subject matter of U.S.  
35 Patent No. 4,945,050 which issued on July 31, 1990 to Sanford et al. Particles e.g., microprojectiles, can be used to transfer genes to a broad range of tissues,

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cells and organelles in many different species. Several acceleration methods such as gun-powder, electric discharge, and gas-power are available to deliver particles through membranes, cell walls, extracellular  
5 matrices, etc. A helium-driven acceleration system has been developed and is described in Sanford et al., Technique 3:3-16 (1991).

The efficiency of gene transfer by physical means and, in particular, by particle bombardment is  
10 greatly improved by using particles encapsulating a dense core which increases cell penetration. Furthermore, the inert, non-toxic substantially pure carbonaceous surface is compatible with the cellular milieu. Because biological substances including nucleic  
15 acids can be associated effectively onto the surface, delivery of such substances into a target is improved.

In another embodiment this invention concerns a method for introducing exogenous nucleic acid into sperm which comprises

20 (a) accelerating a particle having a substantially pure carbonaceous surface and to which surface is associated the exogenous nucleic acid, wherein said particle encapsulates a magnetic core said particle having a diameter sufficiently small to  
25 penetrate the sperm without killing the sperm, and propelling said particles at the sperm whereby said particles penetrate the sperm; and

(b) magnetically selecting the sperm into which the particles have penetrated.

30 The introduction of exogenous nucleic acid into sperm using the method of the invention offers many advantages. Not the least of which is the simplicity of using such sperm for artificial insemination and allowing in vivo development of the embryo. The use of  
35 sperm as vectors for insertion of foreign nucleic acids will facilitate gene integration into the genome.

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The method of the invention is a simple, straight-forward approach for introducing exogenous nucleic acids into sperm. This method constitutes a major advance over the methods currently available because extensive training is not required.

By inserting particles having a substantially pure carbonaceous surface which encapsulates a magnetic core into sperm, a physical means is provided by which to select the sperm containing these particles. Magnetic sorting of sperm after particle bombardment as described in Example 9 below provides a means for enriching the population of sperm carrying the desired exogenous nucleic acids.

In still another embodiment, the method of this invention can be used to make a transgenic animal or non-human mammal which comprises

(a) accelerating a particle having a substantially pure carbonaceous surface and to which surface is associated the exogenous nucleic acid, wherein said particle encapsulates a magnetic core said particle having a diameter sufficiently small to penetrate the sperm without killing the sperm, and propelling said particles at the sperm whereby said particles penetrate the sperm;

(b) magnetically selecting the sperm into which the particles have penetrated;

(c) fertilizing an egg with said magnetically selected sperm in vivo or in vitro;

(d) allowing the product of step (c) to develop to term;

wherein the product of step (d) and/or its progeny is capable of expressing the exogenous nucleic acid.

The introduction of foreign genes into the germ line of animals and the successful expression of the inserted gene are major technological advances in the field of biology. Transgenic animals provide a

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means to produce economically important proteins in milk, blood, eggs, etc. Moreover, such animals can be used to provide tissue for medical transplantation. Transgenic technology offers exciting possibilities for generating precise animal models for human diseases. One commonly used technique for introducing genes into embryos is the direct microinjection of recombinant DNA into the pronucleus of fertilized eggs. This method is tedious, inefficient, requires extensive training and expensive equipment. Such methods require removal of the eggs and in vitro culture of the developing embryo.

Infection of mouse embryos and embryonic stem cells with retrovirus constitutes another method for genetically manipulating embryos. The main disadvantages of the use of retroviruses for gene transfer are the size limitation for transduced DNA and the risk of using retrovirus as a means for gene insertion. Such disadvantages are overcome using the method of the invention.

Techniques for in vitro and in vivo development of fertilized eggs are well known to those skilled in the art.

The following examples are intended to illustrate the invention and are not to be construed as limitations thereon.

#### EXAMPLE 1

##### Particles Having a Substantially Pure Carbonaceous Surface

Nanometer scale carbonaceous polyhedra and tubes were first discovered in 1991, as a by-product of fullerene production as described in Iijima, Nature, vol. 354, pages 56-58 (1991). The apparatus for the synthesis of particles having a substantially pure carbonaceous surface, in the diameter range of 5 to 20 nm, and carbonaceous nanotubes, typically 5 - 30 nm in diameter and 1 - 2  $\mu$ m in length, is very similar to the



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one used for the mass production of C<sub>60</sub> as is described by Kratschmer et al. in Nature, vol. 347, pages 354-358 (1990) and is depicted in Figure 5. It consists of two carbon electrodes (1 and 2), a chamber to maintain the inert gas at a fixed pressure (3), stainless steel rods to position the two electrodes (4), a source of inert gas attached to the chamber (5), a DC current generator (not shown), and a filter and pump arrangement to collect the soot (6). The variable factors are anode (positive electrode) - cathode (negative electrode) gap distance, current at which the arc-discharge experiment is run, voltage (potential difference) between the electrodes, and pressure of inert atmosphere.

The carbon arc-discharge experiments are carried out in a controlled pressure reaction vessel, using a voltage typically in the 18 - 20 volt range. A typical anode (positive electrode) diameter is 6 - 8 mm, and it is usually about 30 cm long.

A typical cathode (negative electrode) diameter is 9 - 13 mm. Cathode diameter is always higher than anode diameter.

Inert gas (either He or Ar) is flowed through the reaction vessel at constant pressure between 100 and 1,000 torr.

The electric current between the electrodes depends on factors such as electrode diameter, gap between electrodes, and inert gas pressure, but can be typically adjusted between 50 and 125 amps.

A computer-controlled motor which adjusts the position of the anode with respect to the cathode is used to obtain a gap distance of 1 mm, and this step initiates the arc-discharge process. During the experiment, the anode is continuously consumed, and a "boule" or "volcano" grows on the cathode. The computer controlled motor ensures that the gap distance between the remaining anode and the tip of the growing boule is maintained at 1 mm.

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The particles having a substantially pure carbonaceous surface are present in the core region of the boule or growth at the end of the cathode. The core region is extracted by means of a sharp pointed tool, and the extract is ground and dispersed in ethanol by ultrasonic means. The dispersed solution is placed on carbon coated electron microscopy grids to facilitate structural analysis. A typical transmission electron microscopic bright-field image is shown in Figure 6, which depicts the morphologies of particles having a substantially pure carbonaceous surface (tubes) (1) and (polyhedra) (2). Figure 1 shows a high resolution image of particles having a substantially pure carbonaceous surface.

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## EXAMPLE 2

## Particles Having a Substantially Pure Carbonaceous Surface Encapsulating A Dense Core

Dense core encapsulated particles having a substantially pure carbonaceous surface in the diameter range of 20 to 50 nm can be produced incorporating electro-positive elements as described in Ruoff et al., Science, vol. 259, pages 346-348 (1993). The apparatus and experimental procedure are similar to those used for processing the particles described in Example 1 above. The following example is shown for  $\alpha$ -lanthanum dicarbide ( $\alpha$ -LaC<sub>2</sub>).

A graphite anode which is 7.9 mm in diameter and 30.5 cm in length is drilled to a depth of 23.0 cm using a 3.2 mm drill.

The cavity in the anode is packed with La<sub>2</sub>O<sub>3</sub>. Weighing of the rod prior to and after packing with La<sub>2</sub>O<sub>3</sub> provides a La:C molar ratio of approximately 0.02.

A graphite cathode is 12.7 mm in diameter. Anode to cathode gap distance is maintained at 1 mm using the computer controlled motor drive for positioning the anode.

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The carbon arc conditions include a DC current of 150 amps, potential difference between anode and cathode of 18 volts, and a helium pressure of 500 torr.

5 A typical high resolution transmission electron microscopic image of  $\alpha$ -LaC<sub>2</sub> encapsulated particle having a substantially pure carbonaceous surface, produced as a result of the arc-discharge experiment using a La<sub>2</sub>O<sub>3</sub> filled anode, is shown in Figure 2.

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### EXAMPLE 3

Particles Having a Substantially Pure Carbonaceous Surface Encapsulating A Dense Core Which Is Magnetic

15 Dense core encapsulated particles having a substantially pure carbonaceous surface with diameters ranging from 20 to 50 nm can be produced where the dense core of the particle is magnetic and amenable to preferential separation using either a permanent or electro-magnet. The apparatus and experimental  
20 procedure are similar to those used for synthesizing any dense core encapsulated particles having a substantially pure carbonaceous surface as described in Example 2 above. The following example is shown for  $\alpha$ -gadolinium dicarbide ( $\alpha$ -GdC<sub>2</sub>), a magnetic material with a Curie  
25 temperature of 293K.

A graphite anode which is 7.9 mm in diameter and 30.5 cm in length is drilled to a depth of 23.0 cm using a 3.2 mm drill. The cavity of the anode is packed with Gd<sub>2</sub>O<sub>3</sub>. Weighing of the rod prior to and after  
30 packing with Gd<sub>2</sub>O<sub>3</sub> provides a Gd:C molar ratio of approximately 0.02. Pure gadolinium (Gd) metal can also be used in the anode. Weighing of the rod prior to and after packing with Gd provides a Gd:C molar ratio of approximately 0.08.

35 Graphite cathode is 12.7 mm in diameter. The anode to cathode gap distance is maintained between 2 and 5 mm with Gd<sub>2</sub>O<sub>3</sub> in the anode using a computer

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controlled motor drive for positioning the anode. With pure Gd in the anode, the anode to cathode gap distance is maintained between 2 and 8 mm. With Gd<sub>2</sub>O<sub>3</sub> in the anode, the carbon arc conditions include a DC current of 75 amps, potential difference between anode and cathode of 18 volts, and a helium pressure of 1,000 torr. With pure Gd in the anode, the carbon arc conditions include a DC current of 75 amps, potential difference between anode and cathode of 18 volts, and a helium pressure of 500 torr.

A typical high resolution transmission electron microscopic image of single crystal  $\alpha$ -GdC<sub>2</sub> encapsulated particles having a substantially pure carbonaceous surface is shown in Figure 3. This particle was produced as a result of the arc-discharge experiment using pure Gd in the graphite anode.

Since  $\alpha$ -GdC<sub>2</sub> is magnetic, particles having a substantially pure carbonaceous surface encapsulating this dense, magnetic core can be preferentially separated using a powerful permanent magnet like samarium cobalt or a high field strength electromagnet at room temperature.

#### EXAMPLE 4

Preparation of Particles With A Substantially Pure Carbonaceous Surface Encapsulating A Dense, Magnetic Core

The method described in this example is based on the methods described in US Patent No. 4,663,230 issued to Tennent on May 5, 1987, the disclosure of which is hereby incorporated by reference.

Catalyst is prepared by placing 10 gm of Davison SMR-37-1534 SRA alumina powder into a 4 oz wide mouth glass jar containing a stirring rod. While stirring the powder, 0.81M Fe(NO<sub>3</sub>)<sub>3</sub> is added dropwise to the point of incipient wetness; 4.1 mL is required. The powder is heated in the jar on a hot plate until dry, the temperature being kept below the level at which NO<sub>x</sub>

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is evolved. The catalyst is then ultrasonically dispersed in water and transferred to a ceramic boat, which is placed in the center of a 1 inch mullite tube in an electric furnace at room temperature. The furnace temperature is raised from room temperature to 500°C over 15 minutes and maintained at 500°C under air for 1 hour. The reactor is then briefly purged with argon at 300 mL/min (flow rates for all of the different gases used in this process, and described below, are the same). The temperature is then raised to 900°C over 15 minutes under hydrogen flow and held at this level for 60 minutes under the same hydrogen flow. The temperature is then raised to 1100°C over 10 minutes, maintaining the same hydrogen flow. Gas flow is then switched to benzene-saturated hydrogen obtained by bubbling hydrogen through benzene at 20°C (approximately 9 parts of hydrogen per part of benzene). Flow is maintained for 10 seconds, and then the gas is switched to argon. The furnace is then cooled to room temperature and the particles having a substantially pure carbonaceous surface are scraped out of the boat.

## EXAMPLE 5

Preparation of DNA-Coated Particles Having a Substantially Pure Carbonaceous Surface Encapsulating a Dense, Magnetic Core

Sixty milligrams of particles having a substantially pure carbonaceous surface encapsulating a dense, magnetic core ( $\alpha$ -GdC<sub>2</sub>) made using the protocol described in Example 3 above are added to a microfuge tube with 100  $\mu$ l of 70 % or 100 % ethanol vortexed for 3-5 minutes and soaked for 15 minutes. The particles are spun in a microfuge for 1 minute. The ethanol is removed and the pellet washed three times in sterile distilled water. At this stage the particles can be stored in sterile 50% glycerol at a concentration of 60 mg/ml. Immediately before use, the mixture is

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vortexed for five minutes and an aliquot of 50  $\mu$ l of the particles is removed.

Plasmid DNA is precipitated onto the particles having a substantially pure carbonaceous surface encapsulating  $\alpha$ -GdC<sub>2</sub> by adding to the mixture 50  $\mu$ l of calcium chloride, 20  $\mu$ l of spermidine (free base; tissue culture grade, Sigma Chemical Co., St. Louis MO.) while vortexing vigorously. Continue vortexing for 2-3 minutes. After incubating for one minute, the particles are pelleted by centrifugation at 12,000 RPM (Sorvall Microspin 12S) for two seconds. The supernatant is removed and discarded. The pellet of particles is washed gently in 70% ethanol and the liquid supernatant is discarded. Again the particles are washed in a 140  $\mu$ l of 100% ethanol, and liquid is discarded. Next 48  $\mu$ l of 100% ethanol is added. The particles having a substantially pure carbonaceous surface are resuspended by vortexing at slow speeds for 2-3 minutes. The ethanol/DNA-coated particle mixture (6  $\mu$ l) is spread evenly onto the center of the 2.4 cm Kapton® disk with the pipette tip. The disks are then dried in a dessicator.

#### RESULTS

The results are shown in Figure 7. DNA - coating on the particles having a substantially pure carbonaceous surface encapsulating a magnetic core are shown using a fluorescent dye, propidium iodide. These particles and their aggregates coat more efficiently with DNA than gold or tungsten particles typically used for particle bombardment and, thus, carry more DNA into the targets.

#### EXAMPLE 6

Particle Acceleration Methods

Particles can be accelerated into the target by a number of mechanisms. These methods include

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centripetal force such as vortexing, and centrifugation, electric discharge, gun powder and compressed air.

#### Particle Delivery System

5           The Biolistic® helium powered PDS-1000/He (Bio Rad, Richmond CA.) is used for these experiments on animal cells in vitro (Figure 8). Gas-shockwaves generated by pressurized helium gas accelerate the particles having a carbonaceous surface and  
10   encapsulating a dense core to high velocities. The particles described, carrying precipitated DNA, are spread onto a thin Kapton® disk (macrocarrier: 2.4 cm in diameter). This disk is held in place about 1 cm from a stopping screen. The helium is retained until the pre-  
15   selected pressure (2000 psi for animal cells) is reached by rupture disks. At this point, the rupture disks (stack of Kapton disks 1.3 cm in diameter) are broken and the gas shockwave propels the macrocarrier containing the DNA-coated particles described above.  
20   The impact of the macrocarrier onto the wire-mesh screen stops the disk but the particles proceed with their DNA-coating into the sample-containing petri dish. Penetration by the particles having a substantially pure carbonaceous surface encapsulating a dense core into the  
25   target (microbes microalgae, plants, animals, tissues, organs, organelles or cells) occurs within the sterile vacuum chamber. Pressure of the helium gas, the level of the tissue within the chamber, and the vacuum pressure within the chamber, control the impact velocity  
30   of the particles.

#### Results

          The size and density of the dense core particles having a substantially pure carbonaceous  
35   surface improves the efficiency of cell penetration with these acceleration methods. The particles having a substantially pure carbonaceous surface encapsulating a

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dense core and to which a biological substance can be associated offer an advantage for penetration of smaller cells and organelles; sperm, microbes, microalgae, nuclei, mitochondria. Moreover the carbonaceous coating improves the association of biological substances to the surface of the particle and their aggregates.

## EXAMPLE 7

Introduction of Exogenous Genetic Substance into Animal Cells

DNA-coated particles are prepared as described in Example 5 above. The plasmid DNA construction used for this experiment and coated on the particles having a substantially pure carbonaceous surface encapsulating a magnetic core was purchased from Pharmacia (Piscataway, NJ). The plasmid (pCH110) consists of the SV-40 promoter and the *lac z* coding region.

Animal Cell Preparation

Throughout the growth and bombardment procedures, the cells were kept under sterile conditions. CHO (Chinese hamster ovary cells) were grown according to established conditions almost to confluency in plastic petri dishes. In general cells are more efficiently transformed while attached to a substrate. CHO cells attach directly on to the plastic dishes. Cells that normally grow in suspension culture are attached for these protocols to the plastic petri dishes with Cell Tak (Collaborative Biomedical Products, Becton Dickinson Labware Bedford MA). Most of the growth medium is removed from the petri dish before placement in the sterilized vacuum chamber.

In Vitro Bombardment

The cells are placed 3 cm from the microcarrier launch assembly. The chamber is evacuated to a level of 15 in. Hg before bombardment. Within



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fifteen minutes after bombardment, the media is replaced, the dish covered and placed in a humidified, CO<sub>2</sub> incubator at temperatures appropriate for the cell type ca. 37°C. Cell Culture / Cell Viability

5 EL4 (T-lymphocyte cells developed from cultures of MRL-lpr/lpr spleen) (Fox et al., J. of Biological Response Modifiers, 9: 499-511 (1990)), and BF-1 (IL2 dependent T-lymphocyte cell from adenocarcinoma) are cultured in 5% CO<sub>2</sub> in RPMI (Gibco) containing 0.1 mM nonessential amino acids, 0.1 M sodium pyruvate, 2mM L-glutamine, and 100 g of gentamicin sulfate. The media is supplemented with 50 m 2-mercaptoethanol (Aldrich) and 10 % fetal bovine serum (Gibco). Growing cells are trypsinized and replated at 10 1 X 10<sup>5</sup> cells per petri dish. Four days after 15 incubation the cultures were ready for bombardment. CHO and EL4 cells attached directly to the plastic dish but the BF1 cells were attached for bombardment with Cell Tak. Cell viability, following bombardment, was 20 estimated using the trypan blue exclusion test.

#### Transient Expression Assays

Histochemical staining to reveal the expression of the marker gene ( $\beta$ -galactosidase 25 (EC3.2.1.23)) is performed 48 hours after bombardment. To detect the  $\beta$ -galactosidase transformed cells, the petri plates are washed three times in Tris-buffered saline. The cells are fixed for 5 minutes in 0.5% (vol/vol) glutaraldehyde in phosphate buffered saline 30 (PBS) washed three times in PBS and then stained at 37°C with X-gal (5-bromo-4-chloro-3-indoyl-6-D-galactopyranoside (Price et al., Proc. Natl. Acad. Sci. (USA), 84: 156-160 (1987)). Transformed cells and clones develop a deep blue stain in 3-6 hours. A dense 35 precipitate appear in these cells after reacting the transferred enzyme with an exogenous substrate and the resulting products catalyze a colored precipitate.

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Control cultures (bombarded without plasmid DNA) do not result in any detectable cellular staining when placed in the enzyme assay solutions. Reporter gene expression efficiencies are determined by counting the number of stained and unstained cells in a hemacytometer. The central 30 mm diameter area of the dish is selected for evaluation.

#### RESULTS

The results are shown in Figure 8. Mammalian cells (Chinese Hamster Ovary cells) show gene expression (blue stain) after bombardment with DNA-coated particles having a substantially pure carbonaceous surface encapsulating a magnetic core. The plasmid DNA containing the coding region for the *lac-z* gene is associated with the surface of these particles.

#### EXAMPLE 8

##### Introduction Of Biological Material Into Sperm By Particle Acceleration

Bovine sperm are prepared by thawing the frozen straws in a water bath (37°C) for 30 seconds. The sperm are then washed twice in sperm-TALP solution (Parrish et al., Biology of Reproduction, 38: 1171-1180 (1988)). Next the sperm are resuspended at  $10 \times 10^6$  sperm per ml in sperm-TALP. Sperm suspensions are enriched for live motile sperm by a "swim-up" protocol (Parrish et al., Theriogenology 25:591-600 (1986)). Sperm motility is then checked under a microscope in order to determine their viability. The sperm are then transiently attached to the surface by placing 200  $\mu$ l of diluted sperm ( $1 \times 10^5$ ) in Cell-Tak coated petri dishes (15mm in size).

Prior to bombardment the particles are coated with the DNA precipitation method of Example 5 above. These particles are dispersed in ethanol and loaded by

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spreading onto a Kapton disk which is allowed to dry in a dessicated area. The particle loaded disk is then placed in the instrument and the appropriate stopping screens and pressure disks are inserted as described in the instruction manual for the Biolistic particle delivery technology (Bio Rad Ca.). Although several Helium pressures, vacuum pressure and target distance levels can be used, higher velocities give better results in penetration of sperm cells. High helium pressures (1500-2500 psi), target distances closer to the acceleration device (3cm) and high vacuum pressure (20 in Hg) are selected for insertion of the particles having a substantially pure carbonaceous surface encapsulating a dense, magnetic core.

15

Sorting Sperm containing Particles Having a Substantially Pure Carbonaceous Surface Encapsulating A Dense, Magnetic Core

After bombardment the sperm are rinsed carefully from the plates, pooled and resuspended in sperm-TALP solution. Next the sperm is washed twice in sperm-TALP (1100 RPM for 10 minutes). In order to separate the live motile sperm from the damaged sperm and particles, the "swim-up" method is used (Parrish et al Theriogenology 25:591-600, 1986). Viable, motile sperm in 1 ml aliquots are gently shaken in a glass tube (Corning 10mm X 75mm). The tube is then placed in an electromagnetic field such that the lower section of the tube is subject to the magnetic force (20,000 Gauss). The fluid in the upper portion of the tube containing sperm-without-magnetic particles is aspirated gently. The remaining sperm-with-particles are resuspended and an aliquot counted and observed under the microscope. The sperm concentration is adjusted to  $25 \times 10^6$  per ml.

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## Results

The results in Figure 9 show mammalian sperm before magnet sorting. Some sperm contain particles with fluorescently labelled DNA and other sperm do not  
5 contain particles with fluorescently labelled DNA. Since the particles encapsulate a magnetic core, sorting with a magnet will separate the sperm containing such particles from those sperm not containing the particles.

10

## EXAMPLE 9

### In Vitro Fertilization With Magnetically Sorted Sperm

#### Oocyte Maturation

The ovaries are collected and stored at 34-  
15 37°C in 0.9% phosphate buffered saline. Next the primary oocytes are aspirated from the follicles (2-8mm) using 18G needle, allowed to settle in a 15 ml tube. Intact cumulus-oocyte complexes are selected and are washed three times in Lb-TALP supplemented with 10%  
20 heat-treated fetal calf serum. Ten cumulus-oocyte complexes are placed in 50 µl droplets of in vitro maturation medium consisting of a 2 ml solution of TCM-199, 10% fetal bovine serum, 20 µg pyruvate, 10µg lutenizing hormone, 1 µg follicle stimulating hormone,  
25 and 5 µl gentamycin under mineral oil. The oocytes are allowed to mature for 20-24 hours in a humidified CO<sub>2</sub> incubator at 39°C (5% CO<sub>2</sub> in air). Next the oocytes are transferred to Fert-TALP (Parrish et al., Biology of Reproduction, 38: 1171-1180 (1988)).

30

#### In Vitro Fertilization

At a concentration of  $25 \times 10^6$ , 2µl of magnetically sorted sperm-containing particles are added to the cumulus-oocyte complexes (10 oocytes) in a volume  
35 of 45 µl droplets. The droplets contain Fert-TALP supplemented with 2 µl heparin and 2 µl of PHE solution which is 10 µM penicillamine, 1 µM hypotaurine, and

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25  $\mu$ M epinephrine. The media and mineral oil (Aldrich Milwaukee WI) are preexposed to the maturation conditions for a minimum of 2 hours. The fertilized cumulus-oocyte complexes are incubated in fertilization media for 12-18 hours at 39°C in a humidified CO<sub>2</sub> incubator (5% CO<sub>2</sub> in air). The resulting zygotes are transferred in in vitro growth droplets (TCM 199, 10% fetal bovine serum, and 50  $\mu$ g/ml gentamycin).

Initial cleavage was assessed 42 hours after adding sperm. Normal development was assessed by counting the number of newly formed two-cell embryos with visible nucleoli.

#### Results

The results presented in Table 1 below show that the fertilization rate with sperm containing particles is assessed at 75% and 66.6% by counting the number of fertilization droplets containing two-cell stage embryos. The percentage of two-cell embryos are 19% and 15% after fertilization with sperm containing particles having a substantially pure carbonaceous surface encapsulating a magnetic core.

Table 1

Fertilization Rate of Sperm with Particles Having a Substantially Pure Carbonaceous Surface

Fertilization Rate	<u>Control</u> Sperm <u>without</u> particles <sup>1</sup>	<u>Experimental</u> Sperm <u>with</u> particles <sup>1</sup>
% Dividing embryos per droplet	66.6%	75%
	100%	66.6%
% Dividing embryos	9%	19%
	43%	15%

<sup>1</sup>Particles having a substantially pure carbonaceous surface encapsulating a magnetic core.

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## Example 10

## Embryo Transfer

- 5           Recipient cows are synchronized in their  
          estrous cycle. Embryos are transferred non-surgically  
          to recipient heifers 5-7 days after estrous day (1-2  
          embryos per uterine horn). Pregnancy is determined by  
          rectal palpation at 45 to 60 days of gestation.
- 10           Artificial Insemination With Sperm Containing DNA-Coated  
          Particles Having a Substantially Pure Carbonaceous  
          Surface Encapsulating a Magnetic Core.
- Recipient cows are artificially inseminated  
15   with magnetically sorted sperm containing DNA-coated  
          particles having a substantially pure carbonaceous  
          surface using procedures as described in Reproduction in  
          Farm Animals (Hafez (ed.)) (1960). Calves are assayed  
          for the transgene, after birth. The casein-hLF gene  
20   (Krimpenfort et al. Bio/Technology 1991) is used for  
          casein signal sequence and flanked by the 5' and 3'  
          untranslated regions of the bovine casein gene. The  
          introns are included since they have been shown to  
          increase expression. The transgene expression is  
25   assayed in the ear tissue of the calf or mammary gland  
          of the adult animals.

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What is claimed is:

1. A method for introducing a biological substance into a target which comprises:
  - 5 (a) associating the biological substance with a particle having a substantially pure carbonaceous surface, said particle having a diameter sufficiently small to penetrate a target without killing the target, and
  - 10 (b) inserting the particle of step (a) into the target.
2. A method according to claim 1 wherein said particle is inserted into the target by accelerating the  
15 particle and propelling the particle at the target whereby said particle penetrates the target.
3. A method according to claim 1 or 2 wherein the biological substance is selected from the group  
20 consisting of nucleic acids, genetic constructs, proteins, toxins, pharmaceutical compounds, hormones, viruses, biological stains, organelles, and vesicles.
4. A method according to claim 1 or 2 wherein  
25 the target is selected from the group consisting of germ cells, microbes, microalgae, plants, organelles, cells, animals, organoids and tissues.
5. A method according to claim 3 wherein the  
30 genetic construct codes for a protein with effective flanking regulatory sequences to express the protein in the target.
6. A method according to claim 3 wherein the  
35 genetic construct is selected from the group consisting of an RNA or a DNA sequence effective to inhibit a native gene or to retard a disease process.

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7. A method according to claim 1 or 2 wherein the particle comprises substantially pure carbon.

5 8. A method according to claim 1 or 2 wherein the particle encapsulates a dense core.

9. A method according to claim 8 wherein the particle encapsulates a metallic core.  
10

10. A method according to claim 9 wherein the particle encapsulates a magnetic core.

11. A method for introducing exogenous  
15 nucleic acid into sperm which comprises  
(a) accelerating a particle having a substantially pure carbonaceous surface and to which surface is associated the exogenous nucleic acid, wherein said particle encapsulates a magnetic core said  
20 particle having a diameter sufficiently small to penetrate the sperm without killing the sperm, and propelling said particles at the sperm whereby said particles penetrate the sperm; and  
(b) magnetically selecting the sperm into  
25 which the particles have penetrated.

12. A method according to claim 11 wherein the exogenous nucleic acid is DNA.

30 13. A method according to claim 11 or 12 wherein the exogenous nucleic acid is a genetic construct coding for a protein with effective flanking regulatory sequences to express the protein.

35 14. A method for making a transgenic animal or non-human mammal which comprises



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- (a) accelerating a particle having a substantially pure carbonaceous surface and to which surface is associated the exogenous nucleic acid, wherein said particle encapsulates a magnetic core said
- 5 particle having a diameter sufficiently small to penetrate the sperm without killing the sperm, and propelling said particles at the sperm whereby said particles penetrate the sperm;
- (b) magnetically selecting the sperm into
- 10 which the particles have penetrated;
- (c) fertilizing an egg with said magnetically selected sperm in vivo or in vitro;
- (d) allowing the product of step (c) to develop to term;
- 15 wherein the product of step (d) and/or its progeny is capable of expressing the exogenous nucleic acid.

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FIG. 1



10 nm

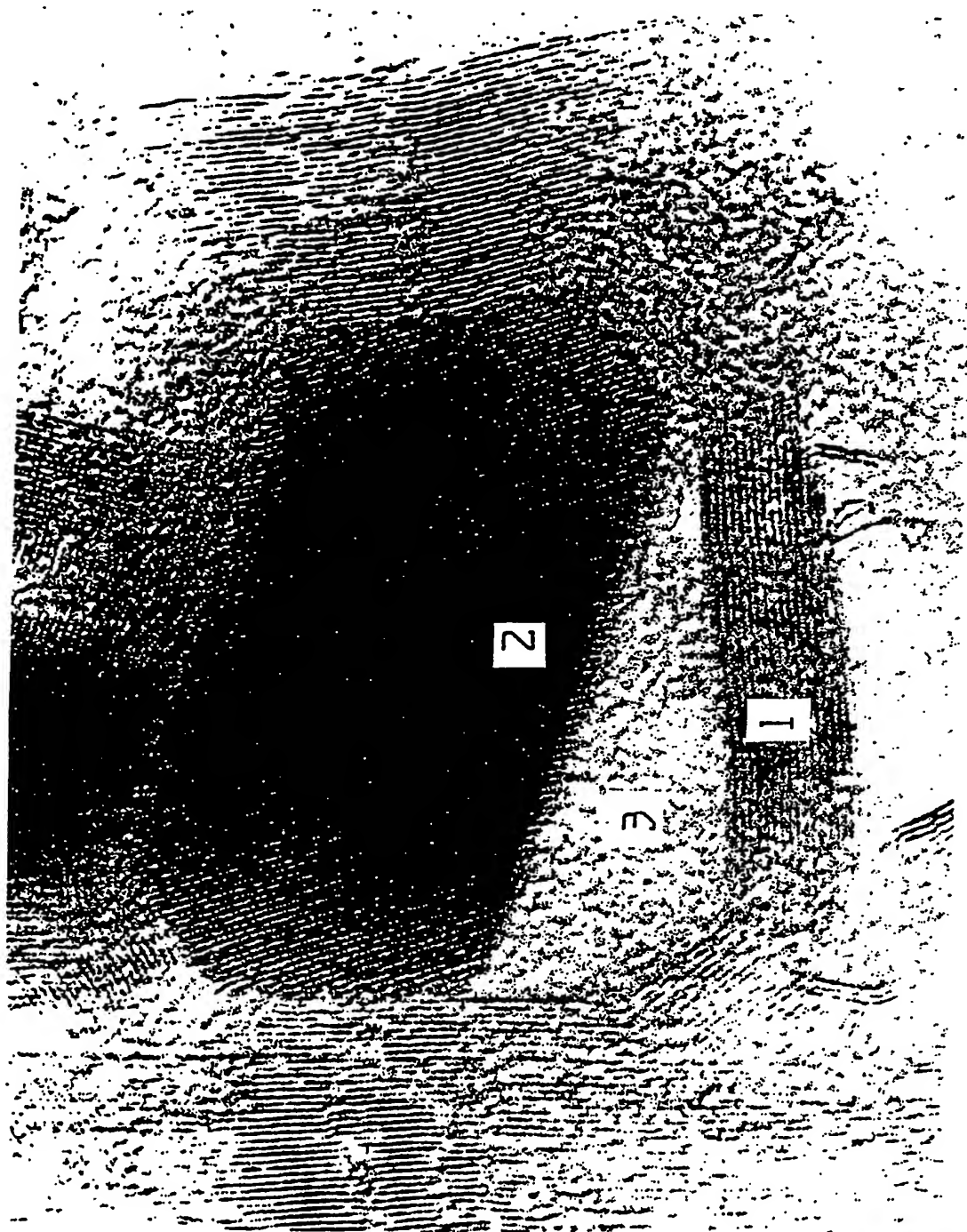
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FIG. 2



10 nm

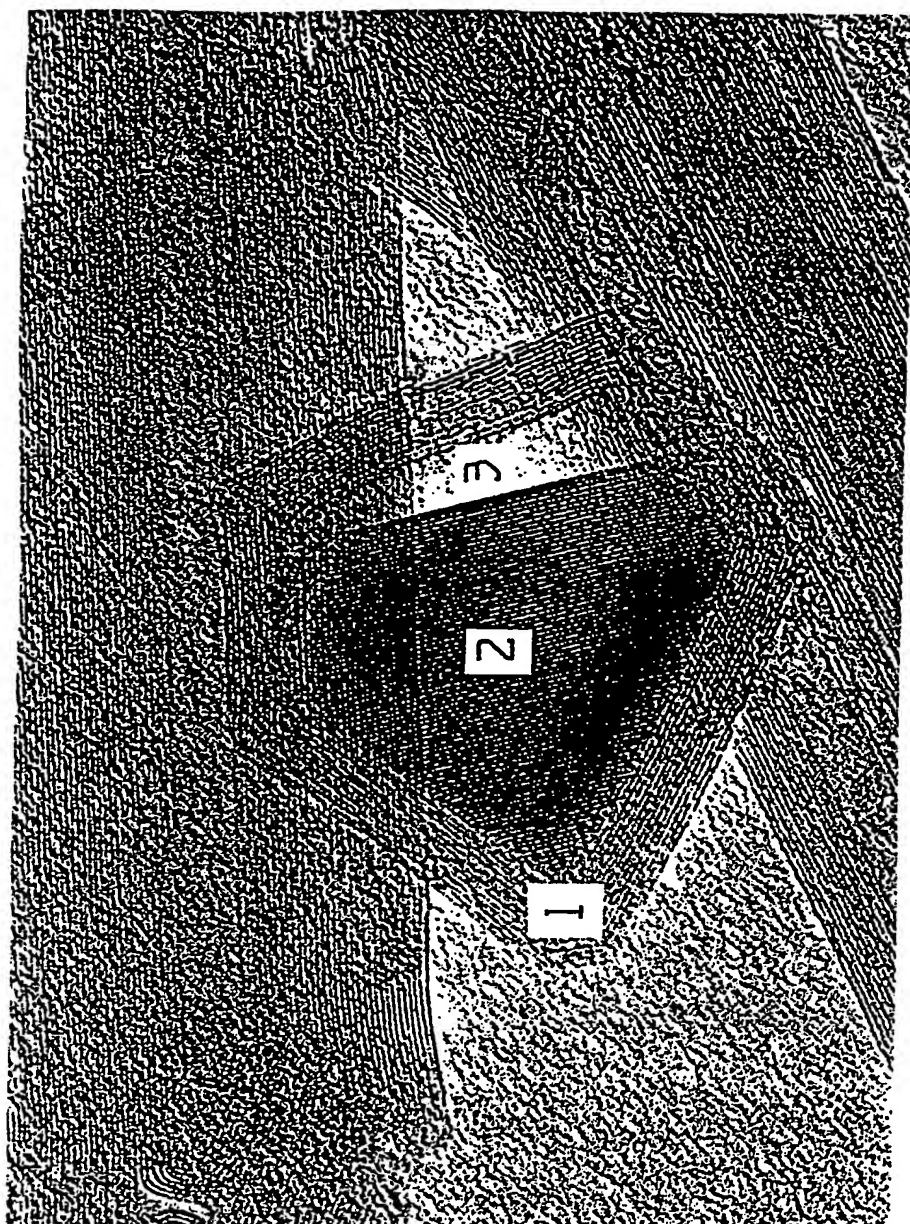
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FIG. 3



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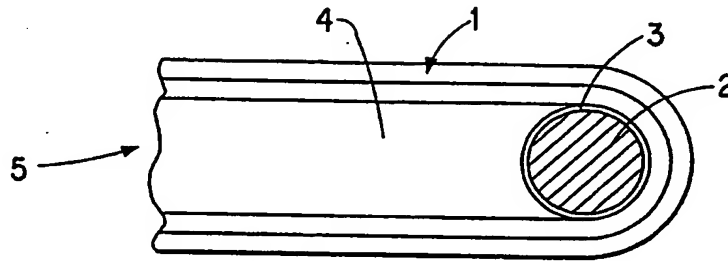


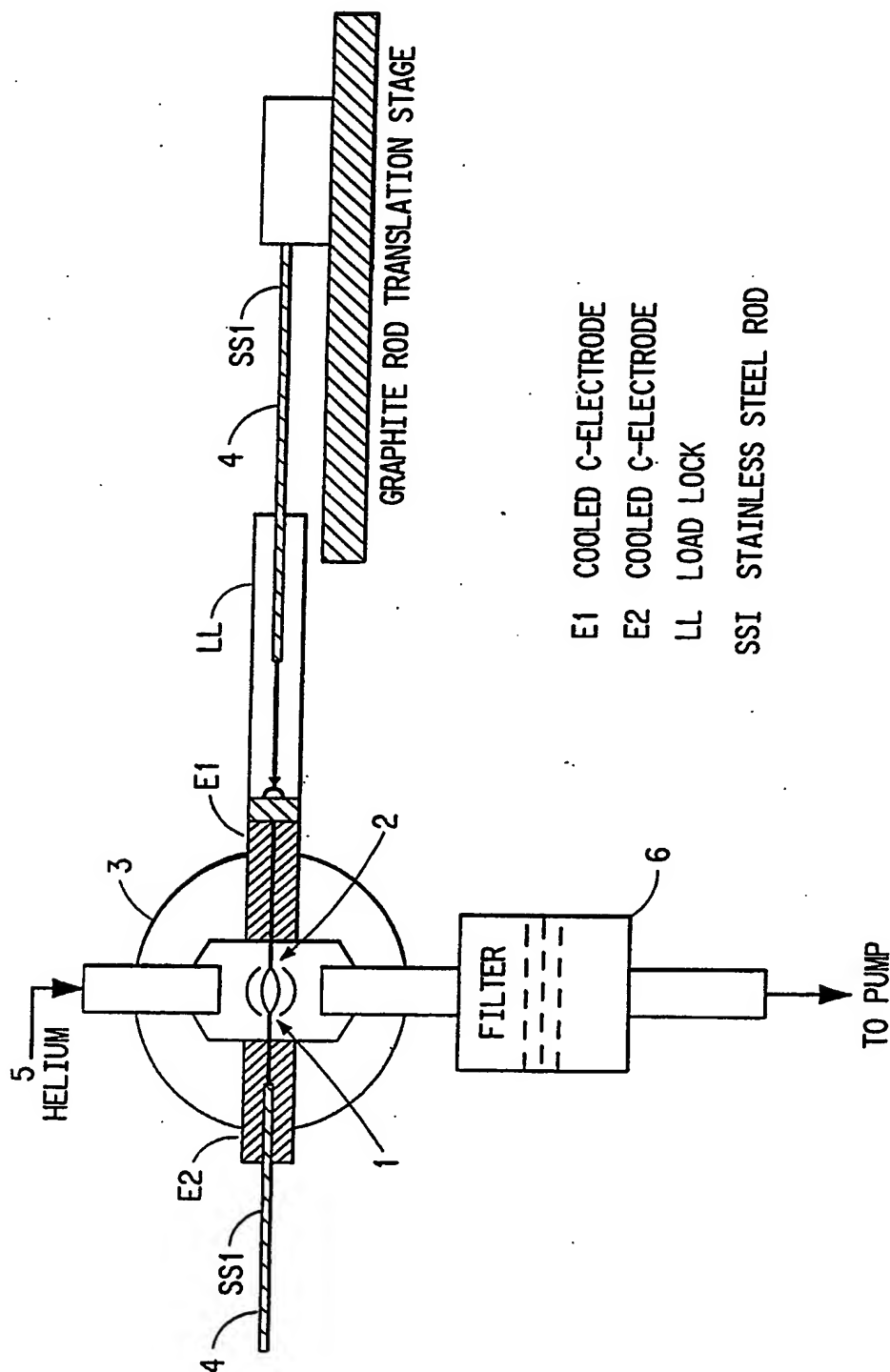
FIG. 4

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FIG. 5



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FIG. 6



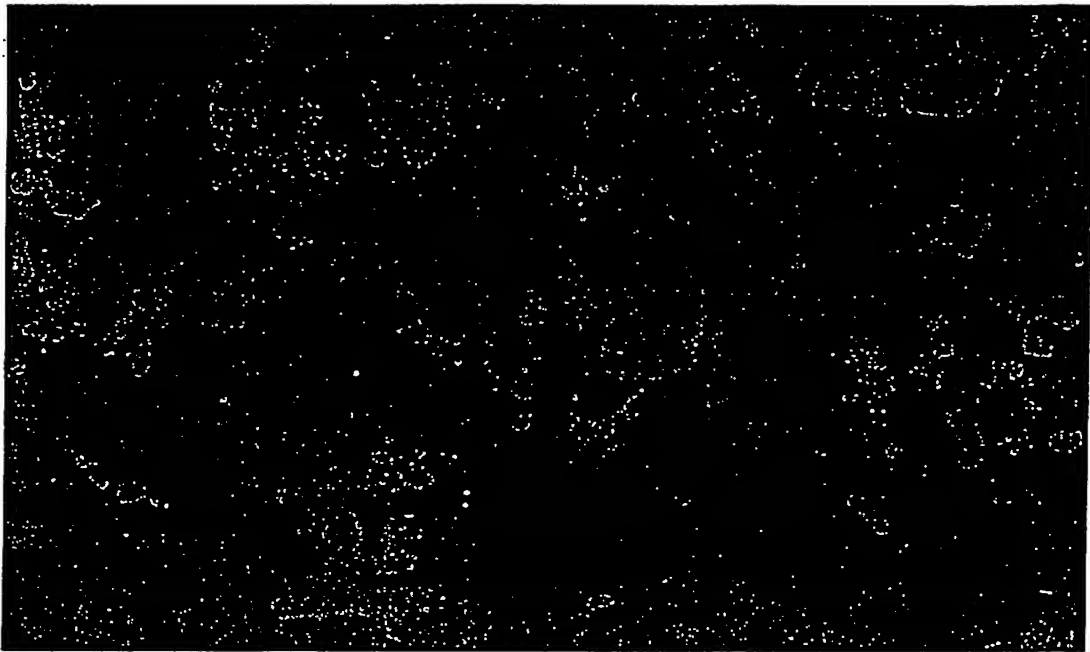
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FIG. 7





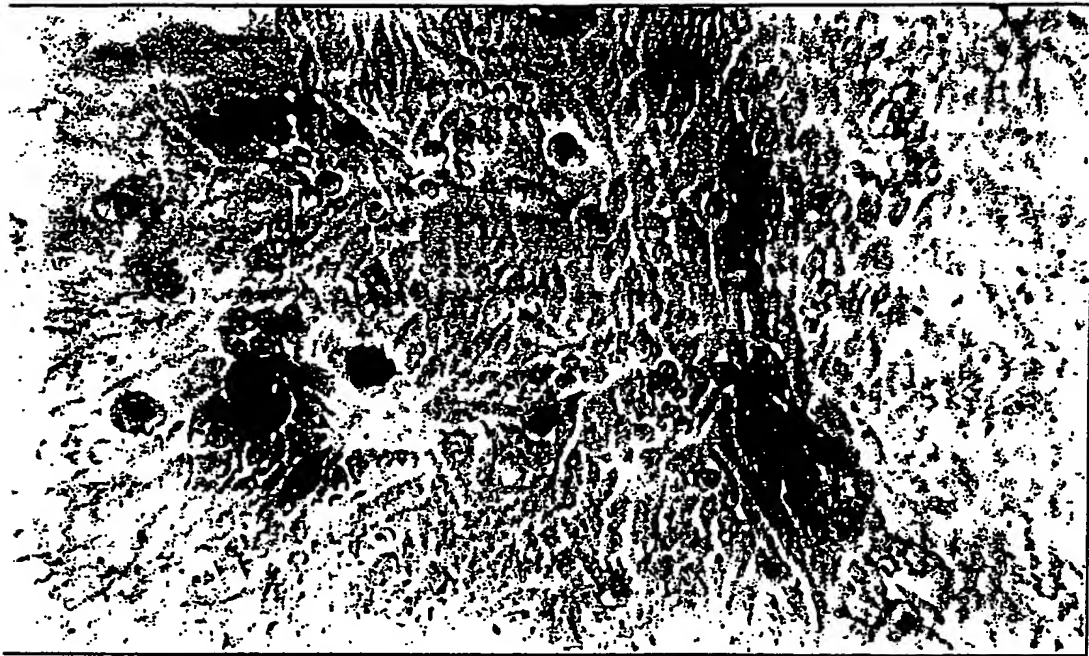
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FIG. 8



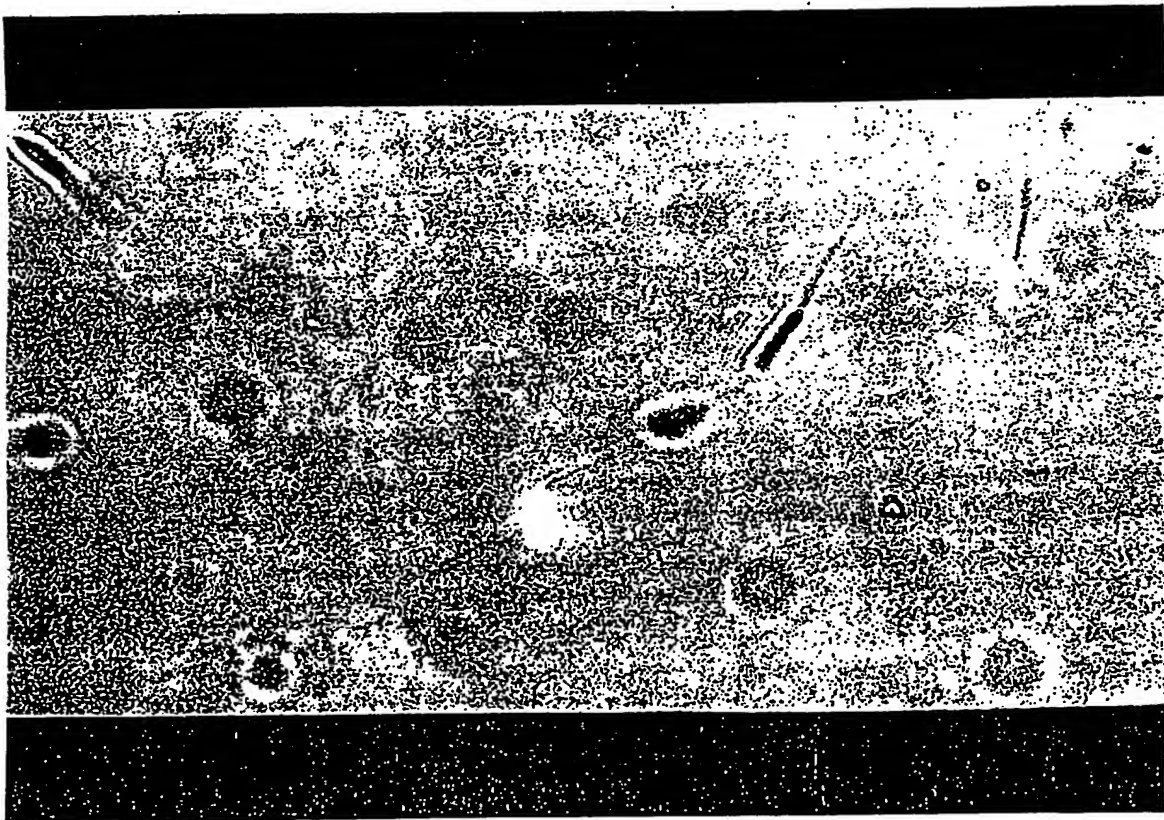
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FIG. 9



## INTERNATIONAL SEARCH REPORT

Inter. Application No  
PCT/US 94/06440

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/87 A01K67/027 C12N15/00 //A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TECHNIQUE, vol.3, no.1, February 1991 pages 3 - 16 SANFORD, J. ET AL. 'An improved, Helium-driven Biolistic device' cited in the application see the whole document ---	1-6
A	WO,A,91 00359 (AGRACETUS, INC.) 10 January 1991 cited in the application see page 3, line 29 - page 4, line 35 see claims --- -/--	1-6, 11-14

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*A\* document member of the same patent family

Date of the actual completion of the international search

6 October 1994

Date of mailing of the international search report

2 1. 10. 94

Name and mailing address of the ISA

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Authorized officer

Andres, S

## INTERNATIONAL SEARCH REPORT

Intern. Application No  
PCT/US 94/06440

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCIENCE (WASHINGTON, D. C.), 15 January 1993 RUOFF, R. ET AL. 'Single crystal metals encapsulated in carbon nanoparticles' cited in the application see the whole document ----	1,7-11
T	CARBON 32(3), 507-13, 1994 SUBRAMONEY, S. ET AL. 'Magnetic separation of GdC <sub>2</sub> encapsulated in carbon nanoparticles' see the whole document -----	1,7-11

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/06440

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark : Although claims 1-14 (as far as in vivo methods are concerned) are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**Information on patent family members**

**PCT/US 94/06440**

Form PCT/ISA/210 (patent family annex) (July 1992)